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Possible Mechanism for Flocculation Interactions Governed by Gene *FLO1* in *Saccharomyces cerevisiae*†

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A model is proposed for the mechanism of flocculation interactions in yeasts in which flocculent cells have a recognition factor which attaches to α-mannan sites on other cells. This factor may be governed by the expression of the single, dominant gene *FLO1*. Isogenic strains of *Saccharomyces cerevisiae*, differing only at *FLO1* and the marker genes *ade1* and *trp1*, were developed to examine the components involved in flocculence. Electron microscopy and concanavalin A-ferritin labeling of aggregated cells showed that extensive and intense interactions between cell wall mannan layers mediated cell aggregation. The components of the mannan layer essential for flocculence were Ca^{2+} ions, α-mannan carbohydrates, and proteins. By studying the divalent cation dependence at various pH values and in the presence of competing monovalent cations, flocculation was found to be Ca^{2+} dependent; however, Mg^{2+} and Mn^{2+} ions substituted for Ca^{2+} under certain conditions. Reversible inhibition of flocculation by concanavalin A and succinylated concanavalin A implicated α-branched mannan carbohydrates as one essential component which alone did not determine the strain specificity of flocculence, since nonflocculent strains interacted with and competed for binding sites on flocculent cells. *FLO1* may govern the expression of a proteinaceous, lectin-like activity, firmly associated with the cell walls of flocculent cells, which bind to the α-mannan carbohydrates of adjoining cells. It was selectively and irreversibly inhibited by proteolysis and reduction of disulfide bonds. The potential of this system as a model for the genetic and biochemical control of cell-cell interactions is discussed.

In some strains of *Saccharomyces cerevisiae*, flocculation or cell agglutination results from distinctive cell-cell interactions. It is controlled primarily by a gene, *FLO1*, which is situated on chromosome I and linked to *ade1* at a distance of 38 centimorgans (40, 44). The phenotypic expression of *FLO1* may be modified by a number of other genes not linked to it (20).

The nature of the interactions among flocculent yeast cells is poorly understood, and basically two models for the mechanism exist. Those based on physicochemical principles propose cooperative hydrogen bonding between cell surface polysaccharides (30) and cross-bridging of anionic functional groups on adjacent cell surfaces by divalent cations, such as Ca^{2+} (30). It is most frequently suggested that cell wall carboxyl groups (30), which may be associated with acidic proteins (2, 22, 45), participate in these chelate complexes. Alternatively, flocculation interactions may be mediated by a specific cell surface recognition mechanism, involving lectin-like binding of surface proteins to polysaccharides on adjacent cells (31, 47).

Lectin-like interactions are fundamental to a variety of biological processes which depend on specific recognition mechanisms (11, 15, 35, 41). The molecular and genetic control of such processes has been best studied in lower eucaryotic model systems, such as the slime mold *Dictyostelium discoideum* (14, 38, 39). The yeast mating types (3, 6, 18, 25, 49) and ciliate mating types (6, 23) provide other, different models. As studies on flocculation interactions are more actively pursued, it may also become a primary model for the genetic control of specific recognition processes. Presently, flocculation has been most studied with brewer's strains of yeasts because of its importance to the brewing industry (13, 43). Unfortunately, brewer's strains are not defined genetically. Although *FLO1* is dominant, its expression may be modified by other genes (20), not all of which are described; therefore, to examine the mechanism of flocculence governed

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by *FLO1*, yeast strains must be carefully selected. The choice of assays and buffers used to measure flocculence is also extremely important since some procedures can result in misinterpretations of data (32). Generally, the objectives and methods used to study flocculence have been fragmented, without consistency in the parameters essential for comparisons of data and a coordinated interpretation of this field of study. In this report, flocculent and nonflocculent strains of a homothallic diploid yeast, *S. cerevisiae* var. *ellipsoideus*, were isogenic and differed only at *FLO1* and the marker genes *ade1* and *trp1*. Under carefully documented conditions, we examined the participation of cations, certain carbohydrates, and proteins in the flocculation interactions, using innovative techniques. With electron microscopy, we illustrated the interactions occurring at the cell surface, and we interpret our findings with a new model for the mechanism of flocculation. Together with other reports from this laboratory (31, 32), we begin a comprehensive examination of flocculence with genetically defined strains of yeast.

MATERIALS AND METHODS

Genetics. The yeast used was *S. cerevisiae* var. *ellipsoideus*. The yeast is homothallic; its haploid spores diploidize shortly after germination to produce diploid spore colonies that are homozygous at all loci other than that for sex. Crosses were performed by mass mating of spore cultures of auxotrophs on plates of minimal medium followed by detection of prototrophs. All other techniques, including tetrad analysis, were routine.

Growth. All yeast strains were grown aerobically in YEPD (1% yeast extract, 2% peptone, 2% dextrose; Difco Laboratories, Detroit, Mich.) by shaking in conical flasks (method B; see reference 32) or in a Microferm fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) with high aeration and vigorous agitation (method A; see reference 32). Growth was monitored by absorbance at 660 nm after the addition of 10 mM EDTA, and logarithmically growing cells were harvested by centrifugation at 940 × *g*. The cell pellets were washed twice with 20 volumes of distilled water. Fresh suspensions of nonflocculent *Schizosaccharomyces pombe* were gifts from G. Calleja of the National Research Council of Canada.

Disruption of cell wall proteins and generation of spheroplasts. Cell wall proteins were disrupted by exposure to mercaptoethanol or proteinase K (E. M. Biochemicals, Darmstadt, West Germany). Washed cells were treated three times with 10% mercaptoethanol–25 mM EDTA–1 mM phenylmethylsulfonyl fluoride–10 mM Tris-hydrochloride, pH 7.0. Each time, cell pellets were resuspended in 2 volumes of the above buffer, incubated at 30°C for 90 min with shaking, and collected by centrifugation at 12,000 × *g*. Cells were also suspended in 2 volumes of 25 mM EDTA–10 mM phosphate buffer (pH 7.0) and digested with 200 μg of proteinase K per g (wet weight) of cells at 30°C with shaking. At the end of various digestion times, cell suspensions were adjusted to 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.). Both mercaptoethanol- and proteinase K-treated cells were washed extensively with distilled water before they were used in other experiments.

Spheroplasts were generated by digestion of the cell wall with zymolyase 60,000 (Kirin Breweries Co., Miyahara, Japan). Cell pellets were resuspended in 2 volumes of 1 M sorbitol–60 mM EDTA–100 mM acetate buffer (pH 7.0) and digested with 200 μg of zymolyase 60,000 per g (wet weight) of cells for 1.0 h at 35°C with shaking. Spheroplasts were collected by centrifugation at 940 × *g* and washed two or three times with 1 M sorbitol.

Settling profiles. Cells were suspended in 2 mM EDTA, pH 8.0, and the cell density was estimated by absorbance at 660 nm, using standard curves. Appropriate cell densities were obtained by dilution, and 0.8 ml of suspension was added to a 1.0-ml cuvette (1-cm path length). Absorbance at 660 nm was measured in a modified Gilford 240 spectrophotometer with a 50-μm-diameter light beam placed 3 mm below the surface of the cell suspension. $CaCl_2$ was added to a final concentration of 5 mM, and the suspension was quickly mixed. Absorbance was monitored automatically with a 2-s dwell. Parallel measurements from four samples were recorded on a calibrated chart recorder. Depending on the requirements of the experiments, the compositions of the solutions and the initial cell densities were altered as described in the figure legends.

Cation and pH dependence. Cells were washed with 2 mM EDTA and resuspended in 2 mM EDTA and 2 mM EDTA with various concentrations of NaCl or KCl. The pH of each suspension was adjusted with HCl or NaOH. Samples of 0.8 ml were adjusted to 5 mM $CaCl_2$, $MgCl_2$, or $MnCl_2$, and settling profiles were determined as above. The density of free cells was estimated as described for the estimation of the critical cell density (32).

Competition experiments. To test for interactions between flocculent and nonflocculent cells, a constant amount of flocculent cells (5×10^7 per ml) was mixed with increasing amounts of nonflocculent cells in the presence of 2 mM EDTA, pH 8.0. The cell suspensions were adjusted to 0.8 ml with the same buffer, and $CaCl_2$ was added to initiate flocculation as above. Once the flocs or cell aggregates had settled, the noninteracting cells in the supernatant were removed and saved. The flocs were washed twice in 5 mM $CaCl_2$ by resuspension and settling and finally dispersed in 0.8 ml of 2 mM EDTA. The numbers of cells per milliliter which had and had not participated in flocculation were estimated separately from the absorbance at 660 nm of the two fractions, after addition of EDTA. The same protocol was followed for interactions between flocculent cells and cells treated with mercaptoethanol or proteinase K. When spheroplasts generated with zymolyase were used, 1 M sorbitol was included in all media. Sorbitol did not inhibit flocculation.

ConA binding. About 6×10^7 cells were suspended in 0.8 ml of phosphate-buffered saline with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. Once flocculation was complete, 80 μg of concanavalin A (ConA)-ferritin (Calbiochem-Behring Corp., La Jolla, Calif.) was added, and the suspensions were incubated at 23°C for 20 min or 2 h with intermittent agitation. The flocs were washed three times with the above solution

and fixed with 5% glutaraldehyde at 4°C. Labeling for 20 min or 2 h appeared to be identical in the amount of ConA-ferritin bound to the cell surface. The specificity of ConA binding was checked by competition with 500 mM α-methyl-D-mannoside (Sigma Chemical Co.). In these experiments the cells were washed twice with 500 mM α-methyl-D-mannoside present in the solution and once without.

For studies on the competitive inhibition of flocculation by lectins, cells were washed once in 2 mM EDTA and twice in phosphate-buffered saline to disperse the flocs. Lectins were added to a final concentration of 1 mg/ml and allowed to bind to the cells for 20 min. Both binding and controls on binding specificity were performed as above. The suspension was adjusted to 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ and further incubated for 20 min. The cells were washed as described above, and settling profiles were determined in the presence of phosphate-buffered saline with cations (32). The cells were recovered and washed once in 2% sodium dodecylsulfate–25 mM EDTA–10 mM phosphate buffer (pH 7.0) and several times in phosphate-buffered saline. Settling profiles were again determined.

ConA (grade IV; Sigma) was succinylated with succinic anhydride (Eastman Organic Chemicals, Rochester, N.Y.; 16). Pea lectin was a gift from M. Young of the National Research Council of Canada.

Electron microscopy. Cells were examined with an electron microscope after they were whole mounted on specimen grids or sectioned. For whole-mount preparation, a sample of cells, about 10^6 cells per ml, suspended in distilled water was placed on Formvar-coated grids, air dried, and further coated with a layer of evaporated carbon. For sectioning, cells were fixed with 5% glutaraldehyde in 0.5 M phosphate buffer or phosphate-buffered saline, pH 6.8 to 7.0, at 4°C for 1.5 h, postfixed with 0.5% (wt/vol) OsO_4 for 30 min, prestained with 1% (wt/vol) uranyl acetate for 1.5 h, dehydrated with ascending series of acetone and then propylene oxide, and finally embedded in Luft's Epon (26) or Spurr's epoxy resin (42). Sections about 90 nm thick were cut with a Dupont diamond knife on an LKB ultramicrotome and stained for 10 min each with 1% uranyl acetate and lead citrate. For ConA-ferritin binding experiments, sections were examined without further contrast staining. Electron micrographs were taken with a Siemens Elmiskop 1A at 80 kV.

RESULTS

Genetics. The *FLO* gene described in this study appeared spontaneously in a strain of yeast that had been subjected to many generations of inbreeding. A genetic analysis of the mutant was carried out with S646, a flocculating strain that was heterozygous for tryptophan and adenine (*trp1*/+ *ade1*/+). This analysis (Table 1) demonstrated that the strain was, in fact, heterozygous for two genes affecting flocculation, *FLO* and a suppressor of flocculation. Crosses between segregants confirmed the presence of a suppressor of varying strength. A strain in which the *FLO* gene was separated from the suppressor was obtained by crossing one of the four segregants of the 0:4 tetrad (Table 1) to a flocculating segregant of a 2:2 tetrad. This strain, S647, produced clear 2+:2− segregation for flocculation. The data of tetrad analysis are summarized in Table 2. It is evident that *FLO* is centromere linked and that it is linked to *ade1*. The two strains S646-1B (*FLO/FLO ade1/ade1*) and S646-8D (*flo/flo trp1/trp1*) were used in subsequent studies.

TABLE 1. Tetrad analysis of a strain S646 (*trp1*/+ *ade1*/+) carrying a spontaneous mutation for flocculation

Marker	+:−		
	2:2	1:3	0:4
Flocculation	4	8	1
Adenine	13	0	0
Tryptophan	13	0	0

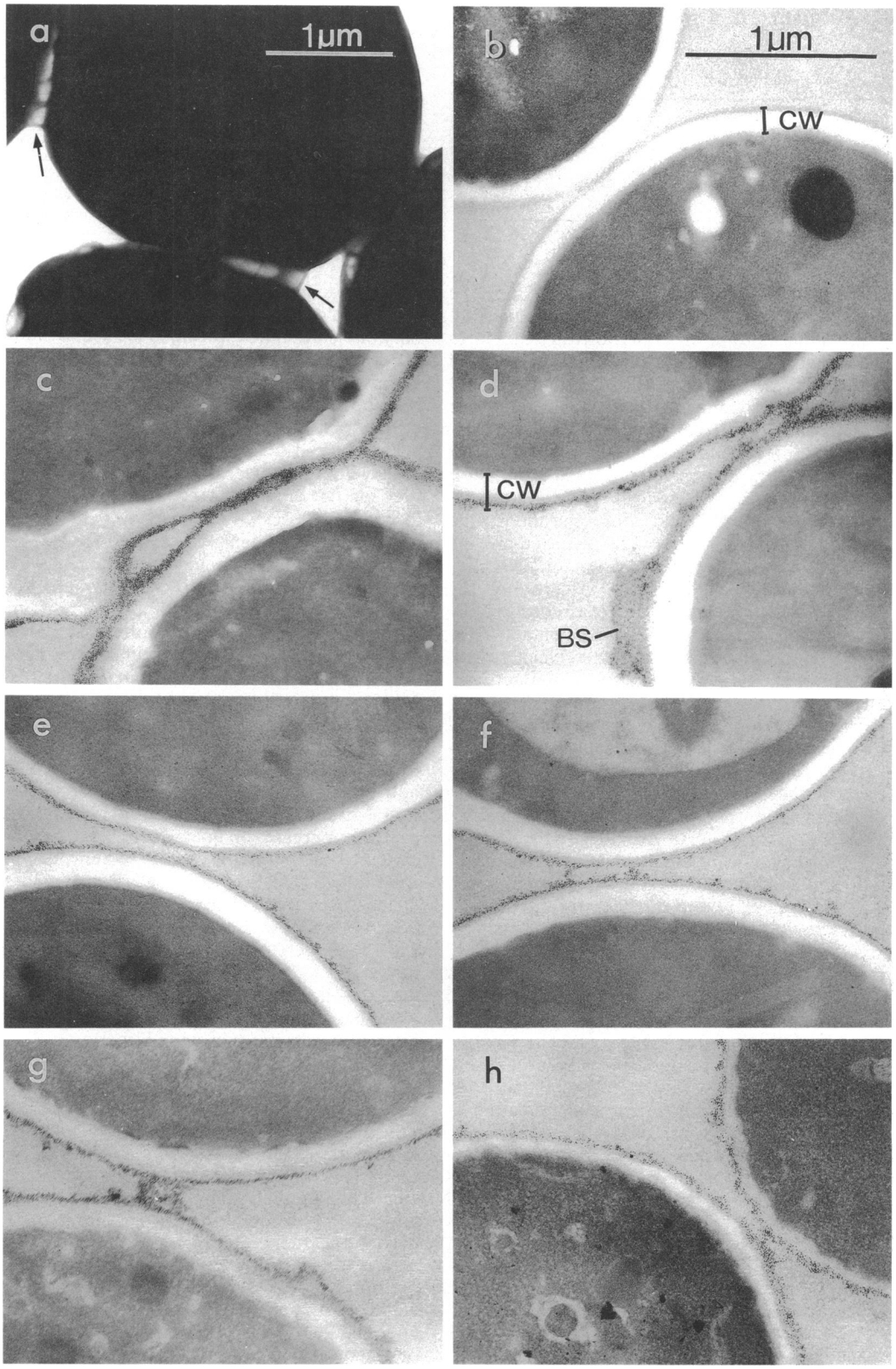
The *FLO* gene was found to be closely linked if not allelic to *FLO1* in the following manner. Strain 1-209 (kindly provided by G. Stewart of Labatts Breweries of Canada Ltd.), containing *FLO1*, was crossed to a nonflocculent homothallic strain by spore-to-cell mating. A homothallic flocculent segregant was selected, and the *FLO1* gene was shown to segregate normally in a series of crosses to nonflocculent strains. Finally, the two *FLO* genes were crossed to produce S648

$$\left(\frac{\text{S619-5B}\ \ \textit{FLO1 met trp}\ \ +}{\text{S647-1B}\ \ \textit{FLO}\ \ +\ \ +\ \ \textit{ade1}}\right)$$

In this strain all of 18 complete tetrads segregated 4+:0 for flocculation but 2+:2− for the three markers.

Microscopy. The cell walls of flocculent and nonflocculent strains were indistinguishable by electron microscopy (Fig. 1). The mannan layer, the outermost layer of the cell wall (21, 24), was selectively revealed by ConA-ferritin binding

FIG. 1. Electron micrographs comparing ConA-ferritin-labeled flocculent (S646-1B) and nonflocculent (S646-8D) strains before and after proteinase K or mercaptoethanol treatment. (a) Whole mount of untreated interacting flocculent cells, showing threadlike connections (arrows) between interacting cell surfaces. (b) Electron micrographs of thin-sectioned nonflocculent cells showing the electron transparency of the cell wall layer (CW) and slight opacity of the surface mannan layer. The cell walls of the flocculent strain were similar. (c) Flocculent and (d) nonflocculent cells with ConA-ferritin bound specifically to the mannan layer. The bud scar (BS) is labeled. (e) Flocculent and (f) nonflocculent cells treated with proteinase K and labeled with ConA-ferritin. (g) Flocculent and (h) nonflocculent cells treated with mercaptoethanol and labeled with ConA-ferritin.


a
1μm
b
1μm
CW
c
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e
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g
h

TABLE 2. Tetrad analysis of S647

$$\left(\frac{\text{S646-1B}}{\text{S646-8D}}\ \frac{FLO\ ade1\ +}{flo\ +\ trp1}\right)$$

Genot.	Parental ditype	Nonparental ditype	Tetratype
ade1–flo1	29	0	29
trp1–flo1	13	16	29
ade1–trp1	28	29	1

TABLE 3. Thickness of ConA-ferritin-bound mannan layers of the flocculent (S646-1B) and nonflocculent (S646-8D) strains after proteinase K or mercaptoethanol treatment

Treatment	Thickness (nm) of mannan layer	
	S646-1B	S646-8D
Proteinase K	31.3 ± 25	32.0 ± 19
Mercaptoethanol	42.6 ± 41	44.6 ± 38
Untreated	51.3 ± 41	48.0 ± 41

(Fig. 1c and d), ConA being a probe for the highly branched α-mannans of *S. cerevisiae* (48). Equivalent extents of ConA-ferritin binding to the surfaces of both strains were observed, and differences in cell wall morphology were not revealed (Fig. 1c and d). During flocculation, the mannan layers of interacting cells merged (Fig. 1a and c). The contact sites between flocculent cells were very extensive (Fig. 1a and c), and it seemed that the entire cell surface had the capacity for interactions. Along the interacting surfaces, the cell walls were distorted by the intensity of the interactions (Fig. 1c). This may have been enhanced by the viscidity of the mannan layer which extended over considerable distances, to maintain cell surface contacts (Fig. 1a). Nonflocculent cells interacted weakly, with very limited extents of cell surface contact (Fig. 1d).

Irreversible inhibition of flocculation resulted from exposure of cells to proteinase K or mercaptoethanol (31). The intense surface interactions between flocculent cells were severely reduced or completely abolished (Fig. 1e and g). The level of ConA-ferritin binding to the mannan layer was significant but reduced in both flocculent and nonflocculent strains. After digestion with proteinase K, the thickness of the mannan layers bound by ConA-ferritin was reduced by about 40% (Table 3; Fig. 1e and f). After reduction with mercaptoethanol, the thickness was not reduced as extensively as with proteinase K; however, a lower density of ConA-ferritin binding was obvious (Table 3; Fig. 1g and h). Characteristically, the mannan surfaces were less uniform after reduction with mercaptoethanol (Fig. 1g and h), perhaps indicating a degradation of the matrix-like structure of this layer (24, 37).

Settling profiles and measurements of flocculence. The progress and extent of flocculation were followed by a decrease in turbidity of a cell suspension on addition of $CaCl_2$. These profiles revealed two components of settling for cells of the flocculent strain S646-1B (Fig. 2). Flocs or cell aggregates settled rapidly past the light beam of the spectrophotometer, resulting in component F; however, free cells that were excluded from the flocs sedimented slowly, resulting in component S. The settling profile for nonflocculent cells of strain S646-8D displayed only one component, owing to the sedimentation of essentially free cells. The intersection of components S and F could be determined by extrapolation and provided an estimate of the critical cell density or the minimum density of cells required for stable flocs to form (31, 32). This value provided a measurement of the degree of flocculation for comparative purposes (32).

Cation requirement. In strain S646-1B, Ca^{2+} was required for maximal flocculence; however, Mg^{2+} and Mn^{2+} may have partially substituted for Ca^{2+} under certain conditions. Flocculation promoted by Mg^{2+} ions was optimal at pH 4 but was not as extensive as flocculation promoted by Ca^{2+} ions (Fig. 3). Above and below pH 4, Mg^{2+} ions were progressively less effective. Mn^{2+} ions also promoted flocculation in a pH-dependent manner, but the degree of flocculation attained was marginal. In sharp contrast to both Mg^{2+} and Mn^{2+}, Ca^{2+}-promoted flocculation was almost independent of pH above 2, but a slight increase in flocculation with pH was evident between pH 3 and 10. When Ca^{2+} ions were added to cell suspensions after Mg^{2+} ions, flocculence was identical to Ca^{2+}-promoted flocculation (Fig. 3). It therefore appeared that Ca^{2+} ions were primarily required for flocculation at pH > 2, whereas at pH 4, Mg^{2+} and other ions may have substituted for Ca^{2+} ions marginally. Nonflocculent cells remained free from interactions under these conditions.

The divalent cation requirement was also studied by competitive inhibition with monovalent cations (Fig. 4). Whereas Ca^{2+}-promoted flocculation was not antagonized by Na^+ or K^+ ions in 100-fold excess over Ca^{2+} ions at pH 4 and 8 (Fig. 4a and b), Mg^{2+}-promoted flocculation was inhibited completely by Na^+ (Fig. 4a) and partially by K^+ ions (Fig. 4b) at pH 4. The partial flocculence promoted by Mg^{2+} at pH 8 (Fig. 4b) was completely inhibited by K^+ ions. It therefore appeared that Ca^{2+} binding to the cells was specific and Mg^{2+} binding was not. Again, nonflocculent cells remained free in parallel experiments. The conditions under which floccula-

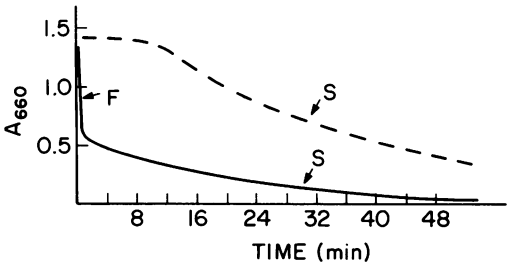

FIG. 2. Settling profiles of flocculent strain S646-1B (—) and nonflocculent strain S646-8D (---). Cells were suspended in 2 mM EDTA, and flocculation was initiated with 5 mM $CaCl_2$. The rapid settling of flocs (F) and slow sedimentation (S) of noninteracting cells were followed spectrophotometrically by the change in absorbance at 660 nm (A_{660}) with time. The intersection of components F and S was estimated by extrapolation and used to estimate the critical cell density (32).

tion was described in this study (pH 8, with or without NaCl) favor Ca^{2+}-promoted flocculation.

Competitive inhibition by succinylated ConA. The participation of α-mannan in the cell-cell interactions was indicated by the competitive inhibition of flocculation by mannose and by ConA (31); however, it is possible that ConA may inhibit cell surface recognition nonspecifically by cross-linking surface receptors. To rule out this possibility, the dimeric form of ConA was used in this study. Previous work (16) showed that when ConA is converted from the tetrameric to the dimeric form by succinylation, both binding efficiency and specificity are maintained but agglutination properties are dramatically reduced. Both succinylated ConA and unmodified ConA inhibited flocculation with the same efficiency (Fig. 5a); however, when binding was competed with α-methyl-D-mannoside, flocculation was unaltered. When bound succinylated ConA was removed from the cell surface with sodium dodecyl sulfate and EDTA, flocculation was recovered (Fig. 5a). Parallel experiments with pea lectin, which also binds to mannosyl and glucosyl residues, did not reveal competitive inhibition of flocculation (Fig. 5b). These results suggested that carbohydrates, in particular those bound by ConA, were involved in flocculation interactions.

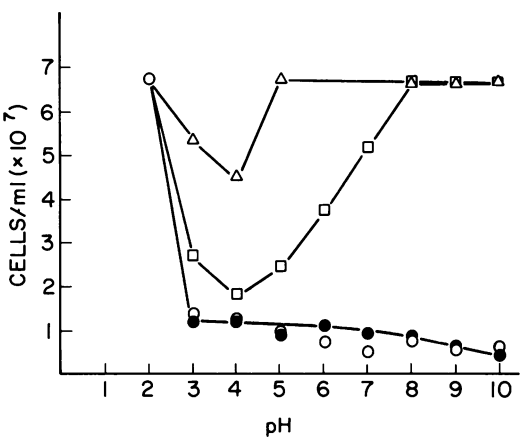

FIG. 3. Cation and pH dependences of flocculation. Cells of the flocculent strain S646-1B were suspended in 2 mM EDTA at various pH values, and flocculation was initiated by the addition of 5 mM $CaCl_2$ (●), 5 mM $MnCl_2$ (△), 5 mM $MgCl_2$ (□), or $MgCl_2$ followed by $CaCl_2$ (○). The critical cell density was estimated as in the legend to Fig. 2 from the intersects of components S and F, which were discernible within 1 to 4 min. The values were plotted against pH.

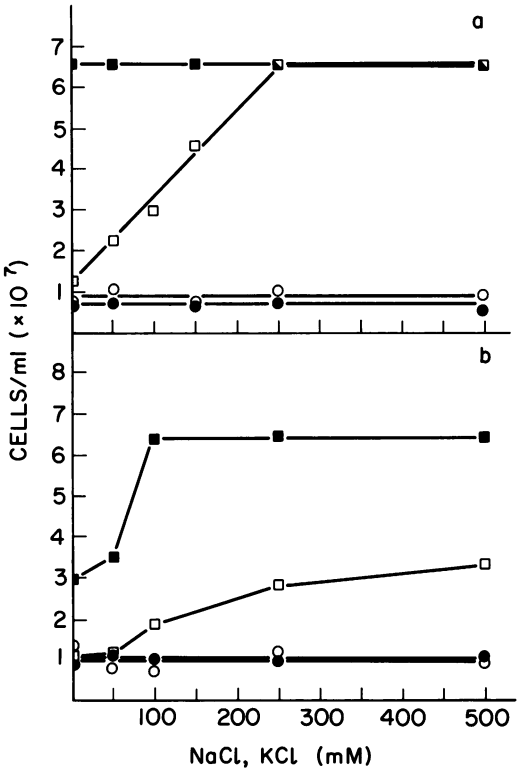

FIG. 4. Competitive inhibition of cation-promoted flocculation by monovalent cations at pH 4 and 8. Cells of the flocculent strain S646-1B were suspended in 2 mM EDTA and various concentrations of (a) NaCl or (b) KCl at pH 4 (open symbols) of pH 8 (closed symbols). Suspensions were adjusted to 5 mM $MgCl_2$ (squares) or 5 mM $CaCl_2$ (circles), and the critical cell densities were estimated as in the legend to Fig. 2 and plotted against the concentration of monovalent cations.

Interactions between flocculent and nonflocculent cells. The mannan carbohydrates bound by

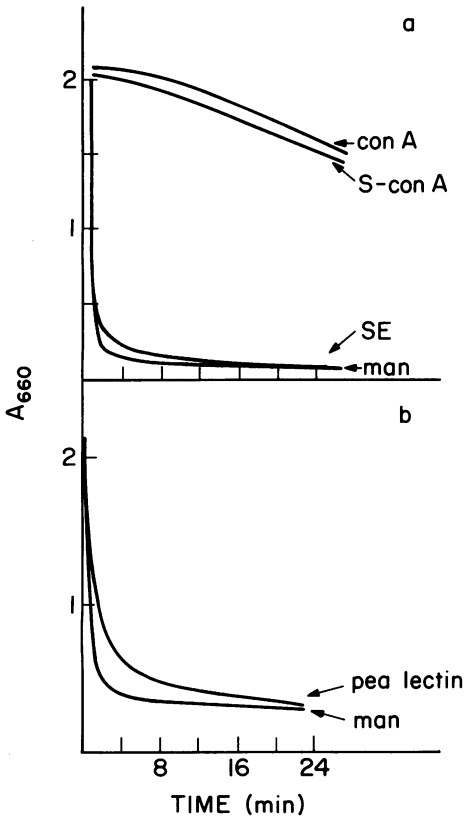

FIG. 5. Competitive inhibition of flocculation by lectins. ConA and succinylated ConA (S-con A) were bound to the surfaces of flocculent cells, and the cells were suspended in phosphate-buffered saline. Lectin binding was competitively inhibited by α-methyl-D-mannoside (man) in parallel experiments, and succinylated ConA, bound to cell surfaces, was subsequently removed with sodium dodecyl sulfate and EDTA (SE). Flocculation was initiated with 5 mM $CaCl_2$, and the settling profiles were determined as in the legend to Fig. 2. (b) Pea lectin was bound to cell walls and binding was also competitively inhibited with α-methyl-D-mannoside. Settling profiles were determined as in the legend to Fig. 5a. A_{660}, Absorbance at 660 nm.

ConA were not strain specific (Fig. 1). If they include recognition and binding sites involved in flocculation interactions, similar sites may also be present on nonflocculent cells. To test this hypothesis, several nonflocculent strains of *S. cerevisiae*, S646-8D, S616-7B-3A, and S598-8A, *Saccharomyces uvarum* 74J, and *S. pombe* were examined in competition experiments. Figure 6a shows that the number of flocculent cells of strain S646-1B which participate in flocculation increased linearly with cell density and the number of cells omitted from the flocs remained constant. When a constant amount of flocculent cells (5×10^7/ml) was mixed with increasing proportions of nonflocculent cells of strain S646-8D, the number of free nonflocculent cells sequestered into flocs increased to a maximum of about two to three cells per flocculent cell (Fig. 6b). As the maximum was attained, the number of free cells increased linearly with cell density (Fig. 6b). When nonflocculent cells were present in great excess, flocculation was dispersive and eventually inhibited. It therefore appeared that sites on nonflocculent cells could compete with sites on flocculent cells for binding. Further-

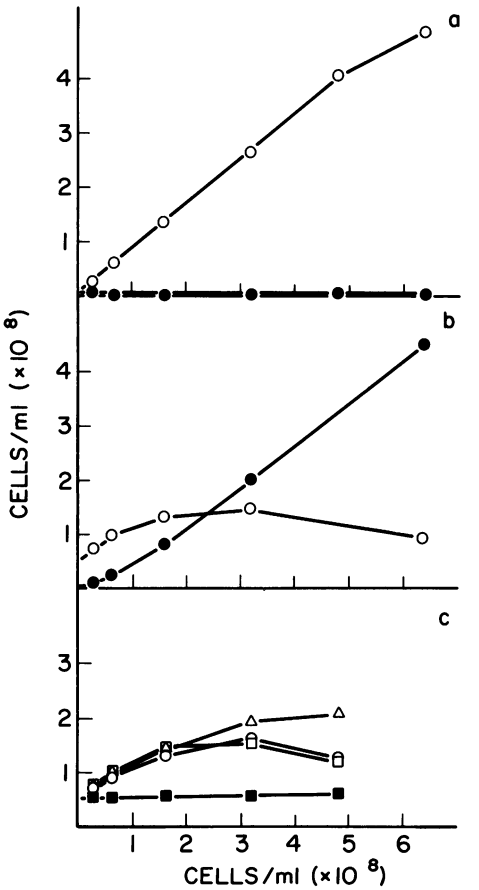

FIG. 6. Interactions between flocculent and nonflocculent cells. (a) Flocculation was initiated with 5 mM $CaCl_2$ in homogeneous dispersed suspensions of flocculent cells of strain S646-1B varying in cell densities (abscissa). The amounts of free cells (●) and cells in flocs (○) were determined by collecting flocs and free cells separately (ordinate). (b) A constant amount of flocculent cells was mixed with various amounts of nonflocculent cells (abscissa) of strain S646-8D and subjected to the above analysis. (c) A constant amount of flocculent cells was mixed with various amounts (abscissa) of other *S. cerevisiae* strains, S616-7B-3A (△) or S598-8A (○), *S. uvarum* strain 74J (□), and *S. pombe* (■). The amount of cells included in flocs was determined (ordinate) as in (b).

more, other strains of *S. cerevisiae* and *S. uvarum* also compete for binding (Fig. 6c); therefore, the recognition and binding sites did not seem to be strain specific and may not be responsible for the strain specificity of flocculation. *S. pombe*, however, did not interact with flocculent cells (Fig. 6c). This finding was significant for two reasons. First, the exclusion of *S. pombe* from flocs verified that nonflocculent cells of *S. cerevisiae* and *S. uvarum* were not nonspecifically trapped into the floc matrix but were interacting with flocculent cells. Second, the inability of *S. pombe* to interact with flocculent cells was correlated with the lack of ConA binding sites on its cell surface (48). These results were consistent with the interpretation that mannan carbohydrates were involved in flocculation interactions, as revealed earlier by the specific competitive inhibition of flocculation by ConA, succinylated ConA (Fig. 5), or mannose (31).

Since the mannan binding sites on various nonflocculent cells were available for interaction, they may act passively in the flocculation interactions. This implies that a separate component unique to flocculent cells exists and actively binds to the mannan carbohydrate when activated by Ca^{2+} ions. Presumably, flocculent cells possess both components. A comparison of Fig. 6a and b was consistent with this interpretation, since the density of flocculent cells limited the extent of the interactions between flocculent and nonflocculent cells. Nonflocculent cells were maximally bound to stable flocs when the ratios of nonflocculent to flocculent cells were 3:1 to 5:1, suggesting that interactions between flocculent cells were preferred over interactions between flocculent and nonflocculent cells. This might occur if the mannan carbohydrates of flocculent cells were more efficiently bound by the active component than those on nonflocculent cells (see below). Alternatively, the interactions between flocculent cells may be bilateral or involve mutual binding of cell surface components, whereas interactions between flocculent and nonflocculent cells may be unilateral (see below).

Selective inhibition of binding activity. Evidence for the existence of two components on flocculent cells which are involved in flocculation interactions was obtained by selective inactivation of the active component. In strain S646-1B, flocculation was very stable to a variety of denaturing conditions but was irreversibly inhibited by digestion of cell walls with zymolyase and by reduction or cleavage of proteins with mercaptoethanol and proteinase K, respectively (31). Removal of cell walls also prevented flocculent cells from interacting with untreated flocculent cells, suggesting the loss of the mannan binding sites as well as the active component (Fig. 7). Disruption of protein structure with mercaptoethanol and proteinase K, however, did not alter the ability of these cells to interact with untreated cells (Fig. 7). Furthermore, the interactions were comparable to the interactions between nonflocculent and flocculent cells shown in Fig. 6 or between untreated flocculent cells and nonflocculent cells treated with mercaptoethanol and proteinase K (31). Figure 1 illustrated residual but extensive binding of ConA to cells exposed to mercaptoethanol and proteinase K. These carbohydrates may have participated in the interactions shown in Fig. 7. The selective loss of the active component, which is proteinaceous or dependent on proteins with disulfide bonds for activity, is implied by the nonflocculent state generated by protein disruption.

DISCUSSION

Flocculation is characterized by intense interactions between yeast cells, which result in the formation of large cell aggregates or flocs. In addition to its importance as an industrially applicable biological trait (13, 17, 43), it also provides a model system for the genetic control of cell surface recognition phenomena. The flocculent and nonflocculent strains developed in this study were isogenic except for the marker genes *ade1* and *trp1* and the gene *FLO1*, which governs flocculation. Genes which suppress the activity of *FLO1* were removed, and the cells were grown under conditions in which flocculation was unaltered during growth (31, 32). The potential exists for genetically manipulating *FLO1* by yeast recombinant DNA techniques, but to interpret such experiments, a greater

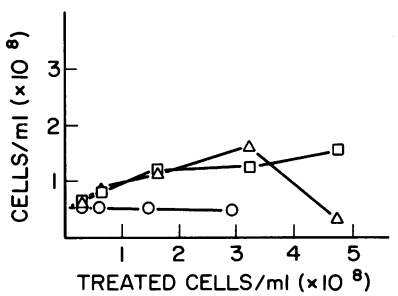

FIG. 7. Interactions between flocculent cells and cells in which flocculation was irreversibly inhibited. Cells of strain S646-1B were treated with proteinase K (□), mercaptoethanol (△), and zymolyase (○) to inhibit flocculation irreversibly (31). Various amounts (abscissa) were mixed with a constant amount of untreated flocculent cells, and the amount of cells recovered in flocs (ordinate) was determined as in the legend to Fig. 6.

understanding of the mechanism and complex control of flocculation is required since critical literature on this subject is scarce.

The participation of cell wall mannan in flocculation interactions was revealed by selective staining of the mannan layer with ConA-ferritin and electron microscopy. Similar observations were made when mannan layers were selectively stained with phosphotungstic acid at low pH (31). Intense interactions extended over large surface areas, resulting in distortions of the cell walls along the contact sites. The stability and intensity of these interactions appear to have been enhanced by the viscidity of the mannan layer. Local differences in cell wall morphology along the contact sites were not evident, as in the mating interactions of **a**- and α-haploid cells (25, 36, 49), and the entire cell surface seemed to have the potential for interaction, thus accounting for the large size of flocs. In previous studies (31), up to five cell contacts per individual cell were visualized in one plane alone. Morphologically, flocculent and nonflocculent cell walls were indistinguishable except for the interactions seen. Surface fibers or fimbriae, which have been implicated in the flocculation of other strains (9, 10) and in the mating interactions between *Hansenula wingei* cells (7), were not revealed in the thin sections shown here, although they have been seen in preliminary whole-mount shadowing preparations (unpublished data).

The mannan layer is viewed as a matrix of glycoprotein associated with several functional glycoproteins such as enzymes (24, 37), sexual agglutinins (3, 6, 7), and possibly lectins involved in flocculation (31). The highly branched mannose homopolymers associated with the mannan layer can provide recognition sites for the binding of antibodies (1), the lectin ConA (48), and endogenous animal cell lectins (15). It is possible that these branched α-mannan structures, in particular those bound by ConA, also act as recognition and binding sites in the flocculation interactions.

The involvement of mannan carbohydrate was indicated by several types of experiments. Flocculation in a variety of *S. cerevisiae* strains is competitively inhibited by mannose specifically (12, 30, 47). This was confirmed in our strain (31); however, up to 500 mM mannose or α-methyl-D-mannoside was required to completely inhibit flocculation. The types of mannan structures participating in flocculation interactions may be the same as, or include, those recognized by ConA. In our studies specific binding of ConA and succinylated ConA to these mannan structures inhibited flocculation, whereas pea lectin did not. ConA-ferritin labeling of the cell surface further showed that structures bound by ConA were densely and evenly distributed over the cell surface, perhaps accounting for the large amount of mannose required for competitive inhibition. Since it is believed that the side chains of the highly branched α-mannan are the recognition and binding sites for ConA (48), they may also be involved in flocculation. Also, selective hydrolysis of phosphomannans with hydrofluoric acid in other strains did not inhibit flocculation or fluorescein isothiocyanate-ConA binding (22). Moreover, we have illustrated that the interactions of a variety of nonflocculent yeasts with flocculent yeast cells were correlated with the presence of surface mannans which bind ConA. Hydrolysis of proteins of nonflocculent cells (31) and flocculent cells did not alter their interactions with untreated flocculent cells, and substantial binding of ConA-ferritin to their cell surfaces was still evident, although reduced in extent. These observations imply the involvement of α-mannan carbohydrates in flocculation but they cannot account for the strain specificity of flocculation as determined by *FLO1*, if it is assumed that the mechanism by which nonflocculent cells bind to flocculent cells also governs the interactions between flocculent cells. In the past, attempts to demonstrate major biochemical differences in cell wall composition have been ambiguous (13, 43), and the cell surface antigenic determinants of flocculent and nonflocculent cells appeared to be very similar (4, 5).

A separate molecular component appears to participate in flocculation interactions and may govern the strain specificity of flocculation. Selective inhibition of this component was achieved by protein reduction and proteolysis, since cells no longer had the capacity to flocculate but could still interact with untreated flocculent cells. It may, therefore, be proteinaceous or at least dependent on proteins with disulfide bonds for activity. Whether this component is the product of *FLO1* or regulated by the product of *FLO1* is not known. Presumably, it is firmly associated with the cell wall since flocculence cannot be removed from our strain by boiling, 6 M urea, 2% Triton X-100, or 2% sodium dodecyl sulfate (31). The stability of this component may depend on its attachment to the cell wall for activity (32). The critical involvement of proteins in flocculation has been recognized in several other studies (33, 34, 45), and differences in the extracts of flocculent and nonflocculent cells have been shown (19, 29); however, the precise manner in which protein functions is not clear.

The binding forces between flocculent cells were suggested to include cooperative hydrogen bonding between cell wall polysaccharides together with Ca^{2+} ion cross-bridging of carboxyl

groups (30) associated with acidic cell wall proteins (2, 45). Cross-bridging of phosphates associated with phosphomannans has also been debated (2, 5, 22, 27, 28). It remains possible that selective Ca^{2+} binding to proteins is mediated by the specific arrangement of carboxyl groups (45); however, Ca^{2+} ions may act as cofactors in activating the binding capacity of certain proteinaceous components to α-mannan carbohydrates rather than as cross-linkers of proteins.

Flocculation has been shown to be dependent on Ca^{2+} ions (30, 31, 46) in extremely minute amounts (46). Yet it has been demonstrated that Mg^{2+} ions also promote flocculation (17). The discrepancy may be due to the pH dependence of Mg^{2+}-promoted flocculation, which differs from Ca^{2+}-promoted flocculation in many ways. Taylor and Orton (46) and Miki et al. (31) assayed flocculence with buffers at pH 7.6 and 8.0, respectively, which are inhibitory for Mg^{2+}-promoted flocculation in our strain but enhance Ca^{2+}-promoted flocculation. Helm et al. (17) assayed flocculence at pH 4.5, which is nearly optimal for Mg^{2+}-promoted flocculation. The results of this study suggest that Ca^{2+} is a primary requirement for flocculation. It is possible that Mg^{2+} substitutes nonspecifically, perhaps by occupying Ca^{2+} binding sites. The results, however, did not dismiss the possibility that separate sites for cation binding and different mechanisms of cell aggregation exist. One mechanism may be Ca^{2+} dependent and independent of pH above pH 3, whereas another, weaker type may be initiated by the binding of Ca^{2+} or other cations optimally at pH 4. The latter may be consistent with one or more of the types of cell aggregation described in the physicochemical models mentioned earlier. In this study, conditions were chosen which optimize Ca^{2+}-dependent flocculation. In the past, the assays and the conditions under which the assays were performed were highly variable and poorly documented with respect to the validity of measurements (32). Furthermore, the strains used were not genetically defined and the expression of *FLO1* or modifiers of *FLO1* was not illustrated; therefore, a detailed comparison of our findings with others may not be meaningful.

The findings of this report are summarized in a model for the mechanism of flocculation (Fig. 8) which is intended as a framework for more detailed studies. *FLO1* may govern the expression of a Ca^{2+}-dependent, proteinaceous, lectin-like component on cell walls of flocculent cells which recognize and adhere to α-mannan carbohydrates on adjoining cells, also recognized by ConA. If the adjoining cell is a flocculent cell, then interactions may be bilateral and very intense (Fig. 8a); however, if the adjoining cell is nonflocculent, then unilateral interactions may occur (Fig. 8b). The viscidity of the mannan layer may stabilize these interactions by permitting extensive and intimate contact. Since the entire cell surface has the potential for interaction, individual cells may interact with several other cells, creating a rigid matrix of cells within the flocs. This model provides one explanation for how a single gene can determine specific binding between two distinct cell wall components. The lectin-like component and the α-mannan carbohydrate conform to the description of cognors and cognons, respectively, which Burke et al. (3) propose as general terminology for the components of recognition systems.

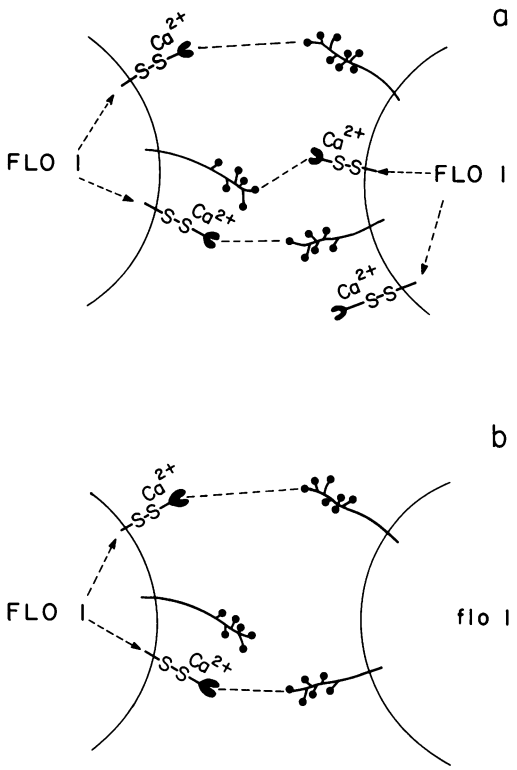

FIG. 8. Model for the possible mechanism of flocculation interactions governed by the gene *FLO1*. Aspects were adapted from the model for sexual agglutination in yeasts (3). (a) Bilateral interactions between flocculent cells. (b) Unilateral interactions between flocculent and nonflocculent cells.

Lectin-like molecules have been implicated in a variety of microbial aggregation phenomena related to life cycle and sexual events; therefore, the concepts of our model are not without precedence. Lectins may be responsible for the cellular aggregations preceding fruiting body formation in the slime mold *Dictyostelium discoi-*

deum (38) and also in the gram-negative bacterium *Myxococcus xanthus* (8). Flocculation, however, may provide a better system for studying the genetic control of cell surface phenomena by lectins, since it is governed by a single gene and is not regulated by complex life cycle events when grown aerobically (31, 32). It can also be utilized as an experimental system since the degree of flocculence can be reproducibly manipulated and induction or repression can be achieved by controlling the aeration of the growth media alone (32). Studies on gene isolation and transformation can also be achieved with the use of the strains developed in this study because *FLO1* is dominant and the strains are homozygous and isogenic, yet possess appropriate marker genes.

The results and interpretations presented here provide a new perspective on flocculation. As studies advance, this system may become a valuable model for studies on cell-cell interactions and contribute to our understanding of the complex functional organization of cell wall structures and biologically active extracellular macromolecules.

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